Detection of Potato Virus X by One Incubation Europium Time-resolved Fluoroimmunoassay and ELISA

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SUMMARY

A time-resolved fluoroimmunoassay (TRFIA) with europium-labelled monoclonal antibodies (MAbs) was used to detect potato virus X (PVX) at a concentration of 5 pg/ml, in potato tuber extracts diluted up to 7 x 10^4-fold and leaf extracts diluted up to 2 x 10^7-fold. When mixed with potato leaf sap, PVX was detected at 100 pg/ml. TRFIA with simultaneous incubation of antigen and labelled antibody (one incubation) was two orders of magnitude more sensitive for PVX detection than the conventional double antibody sandwich ELISA. The fluorescence signal in TRFIA with separate incubations of antigen and labelled antibody (two incubations) with MAbs was linearly related to the virus concentration between 1.6 ng/ml and 1000 ng/ml; the method can therefore be used for the quantification of PVX. PVX detection in plant specimens by one incubation ELISA with MAbs was generally 10 times more sensitive than the standard two incubation procedure. Europium TRFIA with MAbs to potato viruses was found to be a very sensitive method for the detection of PVX, potato virus M, potato virus S, potato virus Y and potato leaf roll virus.

INTRODUCTION

ELISA (Engvall & Perlmann, 1971; van Weemen & Schuurs, 1971) is commonly used to detect a variety of virus antigens in both routine diagnostic and research work. ELISA of plant viruses has a sensitivity in the range of 1 to 10 ng/ml for most viruses (Clark et al., 1986).

Several attempts have been made to improve non-isotopic immunoassay methods, mainly by developing new ways for quantification of immunoreaction products. Although fluorescence measurement is generally a highly sensitive analytical technique, fluoroimmunoassay has not been widely used (Hemmilä, 1985). This is mainly because of the relatively high background fluorescence of biological samples caused by endogenous fluorochromes and light scattering. Time-resolved fluorometry can decrease the background because the excitation of the sample by light pulses and detection of the response fluorescence signal are separated in time. Specialized equipment (Soiri & Kojola, 1983) and europium chelate labels (Hemmilä et al., 1984) have now been developed which enable measurement of Eu concentrations as low as 5 x 10^-14 M. On that basis a time-resolved fluoroimmunoassay using europium-labelled antibodies (EuTRFIA) was developed (Siitari et al., 1983; Hemmilä et al., 1984), which was as sensitive as radioimmunoassay in the detection of some human viruses (Hemmilä, 1985; Halonen et al., 1985). This method has since been applied to the detection of potato viruses (Siitari & Kurppa, 1987; Järvekülg et al., 1987).

In ELISA, reactions take place on a solid phase to which components are sequentially exposed and successively bound. In both direct and indirect ELISA procedures the antigen may simply be adsorbed to the solid phase, or it may be selectively bound by specific antibody or antibody fragments, previously adsorbed to the solid phase. The latter process is widely used in the double antibody sandwich (DAS) procedure, both in ELISA (Clark & Adams, 1977; Clark et al., 1986) and EuTRFIA (Siitari et al., 1983; Hemmilä, 1985). However, for TRFIA a one incubation (OI)
procedure has been described in which simultaneous incubation of antigen and labelled antibody occurs in solution followed by immunosorption on the solid phase-coated antibody (Siitari et al., 1983; Lövgren et al., 1985).

In this study, we investigated the OI procedure because we believed this would be an extremely sensitive method to detect virus antigens, both by EuTRFIA and ELISA. In the latter case the use of the OI procedure is not common, although an analogous ELISA modification has been designed to reduce dissociation of the salt-sensitive particles of apple chlorotic leafspot virus (Flegg & Clark, 1979) and a similar indirect approach is described by Torrance (1980). With OI EuTRFIA, the hepatitis B surface antigen has been detected at 0.2 ng/ml (Siitari et al., 1983) and rotaviruses, adenoviruses and influenza A virus have been assayed with high sensitivity (Halonen et al., 1985).

Since MAbs have been used to improve ELISA for a number of plant viruses (van Regenmortel, 1986), including potato viruses (Gugerli & Fries, 1983; Torrance et al., 1986; Järvekül et al., 1987), we labelled MAbs to potato viruses X (PVX), M (PVM), S (PVS), Y (PVY) and potato leaf roll virus (PLRV) with europium chelate and used them for TRFIA. In this paper we report results obtained with the very sensitive and rapid OI immunoassay method (OI EuTRFIA) developed for detection of PVX with MAbs, and compare the data with the results of OI ELISA, DAS ELISA and two incubation EuTRFIA (TI EuTRFIA, where the antibody and labelled antibody are separately incubated). The results demonstrate the value of EuTRFIA and one incubation procedure for detection of PVX, PVY, PVS, PVM and PLRV.

METHODS

Viruses. PVX was grown in Nicotiana tabacum cv. Xanthi-nc or Datura stramonium L. The leaves of infected plants were harvested 3 to 4 weeks after infection and PVX was purified according to Shalla & Shepard (1970). Virus concentrations were estimated spectrophotometrically using $A_{660\text{nm}}^{\text{nm}} = 2.9$ (Bobkova et al., 1983). Purified preparations of PVX, PVS, PVY and PLRV used in this work were kindly provided by Professor J. G. Atabekov and Dr V. Novikov (Moscow State University).

Antibodies. The isolation of mouse MAbs to PVX was as described elsewhere (Söber et al., 1988). In this study we used MAbs 21XD2, 23XA5 and 21XB4, purified from ascites fluids by precipitation with polyethylene glycol 6000 (PEG 6000) (Söber et al., 1988). Rabbit polyclonal antibodies (PAb) to PVX were isolated and purified by PEG 6000 precipitation as described by Söber et al. (1988). MAbs and PAb were conjugated to horseradish peroxidase (HRP) (Olaine, Riga, U.S.S.R.) using the periodate method (Wilson & Nakane, 1978). Monoclonal antibodies to PVX recognize virus in potato leaves and tubers and work well in DAS ELISA in various combinations and in combination with PAb (Söber et al., 1988). The isolation and characterization of mouse MAbs to PVM, PVS, PVY and PLRV will be published elsewhere (M. Saarma, unpublished results). SDS–PAGE (Laemmli, 1970) showed that more than 90% of the detectable protein in the MAb preparations was immunoglobulin.

Antibody conjugation with europium. A Eu$^{3+}$ chelate of $N^{3}$-(p-isothiocyanatobenzyl)-diethylene triamine-$N^{2},N^{3},N^{5},N^{7}$-tetraacetic acid (LKB Wallac) was used in 30-fold molar excess to antibody for conjugation in phosphate buffer pH 9.5 (Hemmila et al., 1984). Following overnight incubation at 4°C, the conjugates were purified by gel filtration steps, the first on a 1.5 × 15 cm column of Sephade G-50 and the second on a 1.5 × 30 cm column of Sephacryl S-400 (Pharmacia Fine Chemicals). The elution buffer was 0.05 M-Tris-HCl pH 7.7, containing 0.1% NaCl and 0.05% NaN$_3$. The Eu–IgG complex was stored at 4°C.

In order to characterize the conjugates, the Eu fluorescence was measured in an Arcus 1230 Fluorometer (LKB Wallac), after appropriate dilution with enhancement solution (LKB Wallac), and was compared with known EuCl$_3$ standards. The enhancement solution contained 15 μM-2-naphthyltrifluoroacetone, 50 μM-tri-n-octylphosphine oxide and 0.1% Triton X-100 in 0.1 M-acetate-phthalate buffer pH 3.2. The protein concentration was measured spectrophotometrically using $A_{380\text{nm}}^{\text{nm}} = 1.34$, taking into account that the thiourea bond causes an increase in absorbance at 280 nm of about 0.008 units per 1 μmol × 10$^{-7}$ (L. Hemmila, personal communication).

Preparation of plant extracts. Plant material was of two types. Most of the experiments were conducted using potato tubers infected with PVX, PVM, PVS, PVY or PLRV or using leaf samples containing uncharacterized field isolates in potato cv. Ando, Adretta, Sulev, Jõgeva tallane, Alõõ, Mavka or Komsonolets. These infected samples were selected by DAS ELISA and are designated here as 'field samples'. In some experiments, leaves of PVX- and PVM-infected N. tabacum cv. Xanthi-nc were also used and there are designated as 'greenhouse samples'. Healthy potato tuber and field leaf samples were also selected by DAS ELISA. Leaf and tuber samples were extracted by grinding 1 g of leaf or tuber in a mortar and pestle with 2 ml of TBS buffer (20 mM-Tris–HCl, 150 mM-NaCl, pH 7.5), containing 0.05% Tween 20. Tissue from near the stolon end of the tuber was used. For TRFIA and ELISA, sap from healthy or infected tuber and leaf samples was serially diluted in TBS buffer.
Table 1. Characteristics of the europium conjugates of monoclonal antibodies to potato viruses

<table>
<thead>
<tr>
<th>Conjugate designation</th>
<th>Virus antigen</th>
<th>Protein concentration (mg/ml)</th>
<th>Mol Eu per mol IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eu21XD2</td>
<td>PVX</td>
<td>0.22</td>
<td>14.8</td>
</tr>
<tr>
<td>Eu23XA5</td>
<td>PVX</td>
<td>0.15</td>
<td>16.5</td>
</tr>
<tr>
<td>EuY4B1</td>
<td>PVY</td>
<td>0.07</td>
<td>7.1</td>
</tr>
<tr>
<td>EuY3C6</td>
<td>PVY</td>
<td>0.14</td>
<td>20.0</td>
</tr>
<tr>
<td>EuM4C1</td>
<td>PVM</td>
<td>0.20</td>
<td>16.5</td>
</tr>
<tr>
<td>EuM6D5</td>
<td>PVM</td>
<td>0.16</td>
<td>18.6</td>
</tr>
<tr>
<td>EuS4A4</td>
<td>PVS</td>
<td>0.19</td>
<td>14.1</td>
</tr>
<tr>
<td>EuS6C6</td>
<td>PVS</td>
<td>0.20</td>
<td>27.0</td>
</tr>
<tr>
<td>EuL6D5</td>
<td>PLRV</td>
<td>0.18</td>
<td>15.8</td>
</tr>
</tbody>
</table>

**RESULTS**

**Characterization of conjugates**

Conjugates of MAbs to PVX, PVY, PVS, PVM and PLRV with europium chelate or HRP were prepared. The characteristics of the europium conjugates are given in Table 1. The conjugation yielded seven to 27 Eu atoms per IgG molecule, thus giving the conjugates very high specific activity (Ekins, 1985). All the conjugates reacted readily with homologous virus, as shown below; it has been reported that up to 50 Eu atoms can be bound per protein molecule without loss of immunoreactivity and stability (Hemmila et al., 1984).

**Sensitivity of potato virus X detection**

The standard curves for detection of PVX by EuTRFIA with simultaneous incubation of antigen and labelled antibody (OI EuTRFIA) are shown in Fig. 1. When two different MAbs, 21XD2 or 23XA5, were used for coating and Eu–21XD2 used as the tracer, the assay could detect as little as 5 pg/ml of virus, which is two orders of magnitude more sensitive than the detection limit for plant viruses by ELISA using MAbs (Halk & de Boer, 1985). The curve (Fig. 1) is almost linear and continues as such up to 1 µg/ml (data not shown); therefore OI EuTRFIA is suitable for quantification of low concentrations of PVX. The corresponding limit for OI ELISA (i.e. ELISA with simultaneous incubation of antigen and labelled antibody) was 1·6 ng/ml of virus (Fig. 1). Thus OI EuTRFIA, which takes about 1·5 h to carry out, is about 300 times more sensitive than OI ELISA for the detection of PVX and when the incubation time in

**ELISA.** Assays were performed in microtitre plates (Dynatech or Flow Laboratories) and were analysed using a Titertek Multiskan MC eight-channel automatic spectrophotometer (Flow Laboratories). DAS ELISA was carried out using MAbs or PAbs as the coating antibody and HRP-conjugated MAbs as second antibody, as described by Clark & Adams (1977). OI ELISA was performed as the DAS ELISA, with the following modifications. The coated plates were washed twice with TBS, containing 0·05% Tween 20 (TBS-T); 100 ktl specimens in TBS-T were added to the wells and after 5 to 15 min the HRP-conjugated MAbs (100 µl, 1 µg/ml) in TBS-T containing 1% bovine serum albumin were added. There were no differences between the results if either the specimen or the conjugate was added first or if both of them were previously mixed and then added (data not shown). The plates were incubated for 1 to 2 h at 37 °C, washed five times with TBS-T and, from then on, processed as in DAS ELISA.

**EuTRFIA.** Assays were performed in the wells of polystyrene microtitration strips (12 wells/strip; Eflab, Helsinki, Finland) previously coated with the MAbs or PAbs for 18 h at 37 °C in 0·1 M-bicarbonate buffer pH 9·6. Prior to use, the strips were washed twice with TBS-T. In the OI procedure, 50 µl samples (in duplicate) were added into the wells followed in 5 to 15 min by 50 µl of MAb–conjugate, containing 20 ng (if not stated otherwise) IgG in LKB Wallac assay buffer (0·9% NaCl, 0·5% gelatin, 20 µM-diethylenetriamine-N1,N1,N2,N2,N3,N3-pentaacetic acid, 0·01% Tween 40 and 0·05% NaN3 in 0·05 M-Tris–HCl buffer pH 7·75). The strips were incubated for 1 h at 37 °C and washed five times with TBS-T. Following this, 200 µl of the enhancement solution was added to each well and, after shaking for 10 min, the fluorescence in the wells was measured in an Arcus 1230 Fluorometer. The results are presented as counts per second (c.p.s.). For the DAS procedure, 100 µl samples (in duplicate) were added to the strips and incubated for 1 h at 37 °C. After washing twice with TBS-T, 100 µl of Eu–MAb (20 ng) was added to each well. Following further incubation for 1 h at 37 °C, the assay was as described for the OI procedure.
OI EuTRFIA was only 30 min the sensitivity was largely unchanged (Fig. 2). Moreover, the sensitivity achieved with Eu 21XD2 was affected little by using different MAbs or PAbs for the coating (Fig. 2). EuTRFIA using the TI method (separate incubation of antigen and labelled antibody) was 10 times more sensitive than DAS ELISA.

Detection of PVX in plant specimens

PVX in field samples was detectable with OI EuTRFIA in leaf sap or tuber sap diluted up to 70000-fold (Fig. 3). With the OI ELISA, reliable detection of the same samples was possible in leaf sap diluted up to 20000-fold or in tuber diluted up to 2000-fold (Fig. 3). In PVX-infected greenhouse samples virus was detectable in leaf sap diluted up to $2 \times 10^7$-fold (Table 2). When
Table 2. Results of OI FIA for potato viruses in tobacco leaves and infected potatoes using europium-conjugated antibodies*

<table>
<thead>
<tr>
<th>Virus antigen</th>
<th>PVX†</th>
<th>PVM†</th>
<th>PVS‡</th>
<th>PVY‡</th>
<th>PLRV‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating antibody§</td>
<td>PolyX</td>
<td>21XD2</td>
<td>23XA5</td>
<td>PolyM</td>
<td>M6D5</td>
</tr>
<tr>
<td>Tracer</td>
<td>Eu21XD2</td>
<td>EuM6D5</td>
<td>EuS4A4</td>
<td>EuPolyS</td>
<td>EuY3C6</td>
</tr>
<tr>
<td>Sample dilution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1-25 × 10³</td>
<td>391626</td>
<td>1377795</td>
<td>931689</td>
<td>12877</td>
<td>23031</td>
</tr>
<tr>
<td>1:2 × 10⁴</td>
<td>129610</td>
<td>436363</td>
<td>255899</td>
<td>7580</td>
<td>14899</td>
</tr>
<tr>
<td>1:6-25 × 10⁴</td>
<td>35081</td>
<td>102657</td>
<td>62965</td>
<td>3072</td>
<td>3943</td>
</tr>
<tr>
<td>1:2 × 10⁵</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3-13 × 10⁵</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:5-6 × 10⁶</td>
<td>5980</td>
<td>19610</td>
<td>11939</td>
<td>(984)</td>
<td>(1286)</td>
</tr>
<tr>
<td>1:2 × 10⁷</td>
<td>1354</td>
<td>4171</td>
<td>2058</td>
<td></td>
<td>(1272)</td>
</tr>
<tr>
<td>Healthy Control</td>
<td>2137</td>
<td>1565</td>
<td>1864</td>
<td>1997</td>
<td>1714</td>
</tr>
</tbody>
</table>

* The fluorescence detected by one incubation EuTRFIA is presented as c.p.s, and the mean values of duplicate measurements are given. Values below confidence limit are given in parentheses. All sample and control readings include 750 c.p.s, attributable to the enhancement solution.
† PVX and PVM were detected in the virus-infected tobacco leaf samples (greenhouse samples) diluted in tenfold steps in sample buffer.
‡ PVS, PLRV and PVY were detected in the diluted tuber sap of field samples of potato cv. Ando.
§ PAbs are prefixed 'Poly'; all other are MAbs.
| Healthy tobacco leaf sap in PVX and PVM assay; healthy (selected with ELISA) tuber sap cv. Ando in PVY, PVS and PLRV assay.
purified PVX was added to healthy potato leaf sap the detection limit in OI EuTRFIA was 100 pg/ml of PVX (data not shown). Dilution curves obtained with the two incubation procedure are shown in Fig. 4. Two incubation EuTRFIA dilution limits are $1:2 \times 10^4$ with leaf sap and $1:7 \times 10^4$ with tuber sap. Corresponding DAS ELISA values are $1:2 \times 10^3$ and $1:7 \times 10^3$.

In both of the OI EuTRFIA and OI ELISA (Fig. 3) the fluorescence (c.p.s.) or absorbance readings at high virus concentrations were relatively low for the virus concentration tested, and decreased with increasing virus concentration. The same phenomenon was observed with purified virus preparation (data not shown). This hook effect (Lövgren et al., 1985) interferes with the quantification of virus by an OI assay if the concentration is high although qualitative detection is unimpaired.

**Detection of PVY, PVM, PVS and PLRV in plant specimens**

The results of attempts to detect PVY, PVM, PVS and PLRV in leaf and tuber sap by OI EuTRFIA are presented in Table 2. In all cases the method gave sensitive and reliable detection of the viruses.

**DISCUSSION**

For plant virus detection, the limits of sensitivity for direct or indirect ELISA were reported to be around 1 ng/ml (Clark & Adams, 1977). The use of MAbs does not give significant improvement in the sensitivity of ELISA for potato virus detection (Gugerli & Fries, 1983; Torrance et al., 1986; Järvekülg et al., 1987). Recently, Siitari & Kurppa (1987) and our group (Järvekülg et al., 1987) have used EuTRFIA for potato virus detection. Siitari & Kurppa (1987) have shown, with PAbs to PVY and PVX, that the EuTRFIA is five to 100 times more sensitive than DAS ELISA. In TI EuTRFIA, where antigen and labelled antibody are incubated separately, as little as 100 pg/ml of PVX is detected reliably. With MAbs to PVX in OI TRFIA, where antigen and labelled antibody are incubated simultaneously, we were able to detect 5 pg/ml of PVX (Fig. 1 and 2). Thus, with purified PVX the sensitivity limit of the OI EuTRFIA was about 0.5 pg (1.4 x 10^{-20} \text{ mol}) of PVX per well, which is about 8600 virus particles. The corresponding limit for OI ELISA was 1.6 ng/ml of virus while for DAS ELISA it was about 16 ng/ml. Our results show that with purified PVX the TI EuTRFIA is 10 times more sensitive than DAS ELISA and OI EuTRFIA is about 300 times more sensitive than OI ELISA. The cylindrical protein coat of PVX is composed of a tight helical array of 968 identical subunits (Hsu, 1982). The MAb 21XD2, which was conjugated with europium and used as the tracer, recognizes an epitope on the subunit, the epitope being exposed on the virus surface (Radavsky...
Fluoroimmunoassay and ELISA for PVX

et al., 1988; Söber et al., 1988). Thus, in principle, a large number of labelled 21XD2 MAbs may bind to a virus particle, resulting in a substantial increase in fluorescence signal and, therefore, sensitivity. The high sensitivity observed in the OI procedure is probably due to the immune reaction occurring in solution, while in the case of the TI assay one of the immunoreaction components is always immobilized.

The data show that with both EuTRFIA and ELISA the use of an OI procedure for testing plant specimens enables the detection of smaller quantities of PVX in diluted leaf sap than does a TI procedure (Fig. 3 and 4). For tuber specimens there was no difference between the OI and TI methods with EuTRFIA, but DAS was preferable with ELISA. When purified PVX was mixed with healthy potato leaf sap the detection limit for PVX in OI EuTRFIA was 100 pg/ml. Thus, leaf sap interferes with PVX detection. In general, EuTRFIA can detect lower quantities of PVX in plant specimens than ELISA, especially in tuber sap. The EuTRFIA method was superior to ELISA in both its linear range and sensitivity. In quantitative TI EuTRFIA the fluorescence signal was linearly related to the PVX concentration between 1.6 and 1000 ng/ml. In addition to the improved signal to noise ratio in TRFIA, the greater sensitivity of EuTRFIA could also be attributed to the smaller size and higher ‘specific activity’ of the label (Hemmilä et al., 1984; Ekins, 1985). It is important to note that there were no serious problems with background signals in EuTRFIA, using leaf or tuber sap diluted three-fold, which implies that tobacco and potato plants contain little or no fluorescent lanthanides.

The OI method, either in EuTRFIA or ELISA is sensitive, rapid and easy to perform and can therefore be recommended for qualitative large scale routine virus detection in potato plants. To minimize the hook effect in the OI procedure the optimum dilution of specimens must be found. For tuber samples this is 10²-fold and for leaf samples it is 10²-fold or more (Fig. 3).

In addition to PVX, we were able to detect PVM, PVY, PVS and PLRV (Table 1). We conclude that EuTRFIA with MAbs can be a valuable and highly sensitive method for the detection of potato virus and may well be equally useful in the assay of many other viruses.

We wish to thank Dr I. Hemmilä and Dr H. Siitari for the help in Eu labelling and providing the Eu chelate. We are greatly indebted to Professor J. G. Atabekov and Dr V. Novikov for the purified potato virus samples.

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